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(71) Applicant: NEXSTAR PHARMACEUTICALS, INC. [US/US]; Suite 200, 2860 Wilderness Place, Boulder, CO 80301 (US).

(72) Inventors: HEILIG, Joseph, S.; 805 13th Street, Boulder, CO 80302 (US). GOLD, Larry; 1033 5th Street, Boulder, CO 80302 (US).

(74) Agents: SWANSON, Barry, J. et al.; Swanson & Bratschun, L.L.C., Suite 200, 8400 East Prentice Avenue, Englewood, CO 80111 (US).

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(57) Abstract

This invention discloses high–affinity oligonucleotide ligands to complex tissue targets, specifically nucleic acid ligands having the ability to bind to complex tissue targets, and the methods for obtaining such ligands. Tissue targets comprise cells, subcellular components, aggregates or cells, collections of cells, and higher ordered structures. Specifically, nucleic acid ligands to endothelia of the blood brain and CSF–blood barriers are described.

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Systematic Evolution of Ligands by Exponential Enrichment: TISSUE SELEX

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FIELD OF THE INVENTION

Described herein are methods for identifying and preparing nucleic acid ligands to tissues. Tissues are described herein as a collection of macromolecule in a heterogeneous environment. According to this definition, tissues encompass a single cell type, a collection of cell types, an aggregate of cells or an aggregate of macromolecule. The method utilized herein for identifying such nucleic acid ligands is called SELEX, an acronym for Systematic Evolution of Ligands by EXponential enrichment. Specifically disclosed herein are high-affinity nucleic acid ligands which bind to various tissues.

BACKGROUND OF THE INVENTION

A method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules has been developed. This method, Systematic Evolution of Ligands by EXponential enrichment, termed SELEX, is described in United States Patent Application Serial No. 07/536,428, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096, United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Methods for Identifying Nucleic Acid Ligands," now United States Patent No. 5,270,163 (see also WO 91/19813), each of which is herein specifically incorporated by reference. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule.

The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of

randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules, dissociating the nucleic acid-target complexes, amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific, high affinity nucleic acid ligands to the target molecule.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," now abandoned (see also United States Patent No. 5,707,796) describes the use of SELEX in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," now abandoned (see also United States Patent No. 5,763,177) describes a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," now abandoned (see also United States Patent No. 5,580,737) describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, termed Counter-SELEX. United States Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution SELEX," now abandoned (see also United States Patent No. 5,567,588) describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. United States Patent Application Serial No. 07/964,624, filed October 21, 1992, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," now United States Patent No. 5,496,938, describes methods for obtaining improved nucleic acid ligands after SELEX has been performed. United States Patent Application Serial No. 08/400,440, filed March 8, 1995, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chemi-SELEX," now United States Patent No. 5,705,337, describes methods for covalently linking a ligand to its target.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified nucleic acid ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," now abandoned (see also United States Patent No. 5,660,985) that describes oligonucleotide containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent Application Serial No. 08/134,028, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2'-Modified Nucleosides by Intramolecular Nucleophilic Displacement," now United States Patent No. 5,756,703, describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," now United States Patent No. 5,637,459, and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," now United States Patent No. 5,683,867, respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules. Each of the above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

Without question, the SELEX process is very powerful. However, to date the process has been successfully demonstrated primarily with pure, simple targets, such as proteins or small molecules. The present invention provides the first demonstration that complex targets are also compatible with the SELEX process. Tissue SELEX allows one to

obtain nucleic acid ligands to multiple targets simultaneously, and is analogous to performing individual SELEX experiments on all the discrete components of a particular tissue.

It is desirable to be able to obtain nucleic acid ligands to complex tissue targets for various reasons. First, tissue SELEX can be useful to obtain nucleic acid ligands when a distinct target is unknown but a general mode of action of the desired ligand is suggested. Second, tissue SELEX can be useful when nucleic acid ligands are desired based on functional results. Since whole tissues or cells can be used in the SELEX process, it is possible to select for nucleic acid ligands which produce a particular phenotype in the tissue or cell. Third, it can be desirable to obtain nucleic acid ligands to a complex tissue target when it is unclear which single target would be effective. It is also useful to obtain nucleic acid ligands to a complex tissue target if the purified target is unavailable or unstable in its purified form (i.e., a membrane protein). Tissue SELEX allows the potential generation of ligands to previously unknown targets, and may rival monoclonal antibodies as reagents for research, diagnostics and therapeutics.

BRIEF SUMMARY OF THE INVENTION

The present invention includes methods of identifying and producing nucleic acid ligands to complex targets such as tissues and the nucleic acid ligands so identified and produced. More particularly, nucleic acid ligands are provided that are capable of binding specifically to tissues which are macromolecule in a heterogeneous environment, such as whole cells or substructures thereof, aggregates of cells, collections of cells, aggregates of macromolecule and the like.

Further included in this invention is a method of identifying nucleic acid ligands to tissues comprising the steps of (a) preparing a candidate mixture of nucleic acids, (b) partitioning between members of said candidate mixture on the basis of affinity to tissue, and (c) amplifying the selected molecules to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity for binding to tissue. Also included are nucleic acid ligands identified according to such method.

Another embodiment of the invention includes methods wherein a negative selection is performed in order to perfect the discrimination between subtle differences of similar tissue types. In this embodiment, the resulting ligands are specific not only for a

particular tissue type, but can discriminate between subtly different tissues of the same type. For example, this method can discriminate between normal and abnormal tissue types, between induced and uninduced tissue types, etc.

In another embodiment of the invention, a method is provided for identifying previously unknown or uncharacterized epitopes which are components of a larger unknown macromolecule, on the tissue target. The ligands that are evolved by the present invention are capable of binding to previously unknown epitopes and the macromolecule which comprises the unknown epitope can then be identified by standard methods. For example, ligands can be evolved to a previously unknown protein found in the context of a complex tissue target. The ligand of the invention can be used to purify the protein away from the tissue target by standard protein purification and identification methods. These standard methods include affinity purification, microsequencing and cDNA databank searches. In this aspect, the newly identified epitopes which are components of a larger unknown macromolecule, such as new or previously uncharacterized proteins, are provided by the invention. These new epitopes and the macromolecule of which they are a component will be useful as diagnostic and therapeutic agents as well as the ligands that helped identify them.

More specifically, the present invention includes nucleic acid ligands to blood brain barrier tissue and cerebral spinal fluid (CSF)-blood barrier tissue. Also included are nucleic acid ligands to the above-described tissues that are substantially homologous to any of the given ligands and that have substantially the same ability to bind the above-described tissues. Further included in this invention are nucleic acid ligands to the above-described tissues that have substantially the same structural form as the ligands presented herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the procedure for identifying high affinity ligands to molecular targets present in blood brain barrier (BBB) tissue.

Figure 2 illustrates the procedure for identifying high affinity ligands to molecular targets present in choroid plexus tissue.

DETAILED DESCRIPTION OF THE INVENTION

This application describes nucleic acid ligands to complex tissue targets identified generally according to the method known as the SELEX process. As stated earlier, the SELEX technology is described in detail, and incorporated herein by reference, in the SELEX Patent Applications. This method, referred to as the Tissue SELEX process, incorporates complex targets in contrast to the more simple targets previously used in the SELEX process. Certain terms used to describe the invention herein are defined as follows:

"SELEX" methodology refers to the combination of selection of nucleic acid ligands which interact with a target in a desirable manner, for example binding to a protein, with amplification of those selected nucleic acids as described in detail above and in the SELEX Patent Applications. Iterative cycling of the selection/amplification steps allows selection of one or a small number of nucleic acids which interact most strongly with the target from a pool which contains a very large number of nucleic acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved.

"Tissue SELEX" methodology applies the SELEX methodology to tissue targets. Tissue SELEX has several advantages. First, using Tissue SELEX one can obtain ligands to specific cell types in the absence of a defined understanding of the involved epitope. The epitope against which a ligand is evolved is usually a substructural component of a larger macromolecule. The ligands found by this method could also be useful in identifying new proteins or other new macromolecules on the tissue target. The new proteins or other new macromolecules which comprise a newly identified epitope can be purified and characterized using standard procedures. Second, ligands can be obtained to defined epitopes or macromolecules in the context of their physiologic cellular or membrane environment. Examples of various tissue targets can include a membrane protein on a whole cell, a plasma protein in plasma, a nuclear protein in the presence of whole nuclear extracts, etc. Third, it is possible to obtain ligands to tissues in a functionally altered phenotype, e.g., activated, migrating, etc. The ligands and the new macromolecule containing the ligand epitopes identified by this process may be useful as diagnostics or therapeutics. Fourth, Tissue SELEX is a powerful methodology which allows one to identify nucleic acid ligands that can mediate many different cell behaviors, such as apoptosis, anergy, differentiation, proliferation, etc., without prior knowledge of the identity of the specific tissue targets that control these changes. The sensitivity of the SELEX process may lead to the generation of

oligonucleotides that recognize potentially every different epitope on the complex tissue target. Larger numbers of different sequence motifs are expected using the tissue SELEX process, as compared with simple-target SELEX, since it is believed that different motifs will recognize distinct epitopes on the complex tissue target. Some epitopes may lie within the same protein, but many will be directed to various proteins or other molecules on the tissue. Tissue SELEX can be done *in vivo* or *in vitro*.

Tissue SELEX allows one to work with a complete living "element" (a cell or bigger) that allows one to *phenotypically* screen for a target-ligand interaction that effects this "element." For example, one could screen an evolved, high affinity tissue SELEX pool using flow cytometry for sequences which bind a membrane protein and cause the cell to carry out a biochemical transformation which is measured by the flow instrument.

Tissue SELEX allows one to obtain nucleic acid ligands to multiple targets simultaneously. All independent binding sites on a very large macromolecular complex such as a tissue or cell should be potential targets for selection. In effect, this allows one to take a tissue and carry out numerous SELEX procedures on this tissue that is theoretically equivalent to individual SELEXes on all individual components of the particular tissue.

In one embodiment, a negative selection process (termed counter-SELEX) is employed to enhance the possibility that the ligands derived by tissue SELEX have precise specificity and affinity. In this embodiment, ligands are selected for a specific tissue and then a negative selection is done against a related tissue which does not have certain characteristics for which the ligand is desired. The negative selection can be done against a similar cell line or cell type, different cells, normal tissue, plasma or blood, a non-specific antibody or other available ligand. An example of negative selection would be to first select using a tumor cell target (such as a malignant melanoma) and then counterselect the resulting nucleic acids against a similar cell type which is not tumorogenic (such as normal human melanocytes). Ligands that interact with both normal and neoplastic tissue will be removed by this negative selection and only those nucleic acid ligands that specifically bind the tumor cells will be identified (or retained). The resulting nucleic acid ligand(s) would be specific for tumors. This technique will provide the ability to identify nucleic acid ligands that can discriminate between two closely related targets, i.e., between a cancerous cell and an untransformed cell of the same tissue type. The negative selection can also be done *in vivo*. Using this method one can not only generate ligands to specific targets on complex

tissue surfaces, but also be able to recognize the differences between normal and abnormal tissue of a particular type.

"SELEX Target" or "Target" refers to any compound upon which a nucleic acid can act in a predetermined desirable manner. A SELEX target molecule can be a protein, peptide, nucleic acid, carbohydrate, lipid, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, pathogen, toxic substance, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, etc., without limitation. Virtually any chemical or biological effector would be a suitable SELEX target. Molecules of any size can serve as SELEX targets. A target can also be modified in certain ways to enhance the likelihood of an interaction between the target and the nucleic acid.

"Tissue target" or "Tissue" refers to a certain subset of the SELEX targets described above. According to this definition, tissues are macromolecules in a heterogeneous environment. As used herein, tissue refers to a single cell type, a collection of cell types, an aggregate of cells, or an aggregate of macromolecules. This differs from simpler SELEX targets which are typically isolated soluble molecules, such as proteins. In the preferred embodiment, tissues are insoluble macromolecules which are orders of magnitude larger than simpler SELEX targets. Tissues are complex targets made up of numerous macromolecules, each macromolecule having numerous potential epitopes. The different macromolecules which comprise the numerous epitopes can be proteins, lipids, carbohydrates, etc., or combinations thereof. Tissues are generally a physical array of macromolecules that can be either fluid or rigid, both in terms of structure and composition. Extracellular matrix is an example of a more rigid tissue, both structurally and compositionally, while a membrane bilayer is more fluid in structure and composition. Tissues are generally not soluble and remain in solid phase, and thus partitioning can be accomplished relatively easily. Tissue includes, but is not limited to, an aggregate of cells usually of a particular kind together with their intercellular substance that form one of the structural materials commonly used to denote the general cellular fabric of a given organ, e.g., kidney tissue, brain tissue. The four general classes of tissues are epithelial tissue, connective tissue, nerve tissue, and muscle tissue.

Examples of tissues which fall within this definition include, but are not limited to, heterogeneous aggregates of macromolecules, such as fibrin clots which are acellular; homogeneous or heterogeneous aggregates of cells; higher ordered structures

containing cells which have a specific function, such as organs, tumors, lymph nodes, arteries, etc.; and individual cells. Tissues or cells can be in their natural environment, isolated, or in tissue culture. The tissue can be intact or modified. The modification can include numerous changes such as transformation, transfection, activation, and substructure isolation, e.g., cell membranes, cell nuclei, cell organelles, etc.

Sources of the tissue, cell or subcellular structures can be obtained from prokaryotes as well as eukaryotes. This includes human, animal, plant, bacterial, fungal and viral structures.

"Nucleic acid" means either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the individual nucleic acid bases or to the nucleic acid as a whole. Such modifications include, but are not limited to, modified bases such as 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, substitution of 5-bromo-uracil; backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping. Modifications that occur after each round of amplification are also compatible with this invention. Post-amplification modifications can be reversibly or irreversibly added after each round of amplification. Virtually any modification of the nucleic acid is contemplated by this invention.

"Nucleic acid test mixture" or "nucleic acid candidate mixture" is a mixture of nucleic acids of differing, randomized sequence. The source of a "nucleic acid test mixture" can be from naturally-occurring nucleic acids or fragments thereof, chemically synthesized nucleic acids, enzymatically synthesized nucleic acids or nucleic acids made by a combination of the foregoing techniques. In a preferred embodiment, each nucleic acid has fixed sequences surrounding a randomized region to facilitate the amplification process. The length of the randomized section of the nucleic acid is generally between 8 and 250 nucleotides, preferably between 8 and 60 nucleotides.

"Nucleic acid ligand" is a nucleic acid which has been isolated from the nucleic acid candidate mixture that acts on a target in a desirable manner. Examples of actions on a target in a desirable manner include, but are not limited to binding of the target,

actions on a target in a desirable manner include, but are not limited to binding of the target, catalytically changing the target, reacting with the target in a way which modifies/alters the target or the functional activity of the target, covalently attaching to the target as in a suicide inhibitor, facilitating the reaction between the target and another molecule. In most, but not all, instances this desirable manner is binding to the target. In the most preferred embodiment, a nucleic acid ligand is a non-naturally occurring nucleic acid ligand having a specific binding affinity for a tissue target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to said nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein said nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule. Nucleic acid ligand includes nucleic acid sequences that are substantially homologous to the nucleic acid ligands actually isolated by the Tissue SELEX procedures. By substantially homologous, it is meant a degree of primary sequence homology in excess of 70%, most preferably in excess of 80%, and even more preferably in excess of 90%, 95%, or 99%. The percentage of homology as described herein is calculated as the percentage of nucleotides found in the smaller of the two sequences which align with identical nucleotide residues in the sequence being compared when 1 gap in a length of 10 nucleotides may be introduced to assist in that alignment.

In the past it has been shown that various nucleic acid ligands to a specific target with little or no primary homology may have substantially the same ability to bind the target. For this reason, this invention also includes nucleic acid ligands that have substantially the same ability to bind a target as the nucleic acid ligands identified by the Tissue SELEX process. Substantially the same ability to bind a target means that the affinity is within a few orders of magnitude of the affinity of the ligands described herein. It is well within the skill of those of ordinary skill in the art to determine whether a given sequence -- substantially homologous to those specifically described herein -- has substantially the same ability to bind a tissue target.

The invention also includes nucleic acid ligands that have substantially the same postulated structure or structural motifs. Substantially the same structure or structural motifs can be postulated by sequence alignment using the Zukerfold program (see Zuker (1989) Science 244:48-52). As would be known in the art, other computer programs can be used for predicting secondary structure and structural motifs. Substantially the same structure

or structural motif of nucleic acid ligands in solution or as a bound structure can also be postulated using NMR or other techniques as would be known in the art.

"Partitioning" means any process for separating nucleic acid ligands from the remainder of the unreacted nucleic acid candidate mixture. Partitioning can be accomplished by various methods known in the art. Filter binding, affinity chromatography, liquid-liquid partitioning, filtration, gel shift, density gradient centrifugation are all examples of suitable partitioning methods. Equilibrium partitioning methods can also be used as described in detail below. Since the tissue targets of the present invention are non-soluble, there are numerous simple partitioning methods which are well suited to this invention. The simple partitioning methods include any method for separating a solid from a liquid, such as, centrifugation with and without oils, membrane separations and simply washing the insoluble tissue target. The ligands can also be specifically eluted from the target with a specific antibody or ligand. The choice of partitioning method will depend on properties of the target and the nucleic acid and can be made according to principles and properties known to those of ordinary skill in the art.

"Amplifying" means any process or combination of process steps that increases the amount or number of copies of a molecule or class of molecules. In preferred embodiments, amplification occurs after members of the test mixture have been partitioned, and it is the facilitating nucleic acid associated with a desirable product that is amplified. For example, amplifying RNA molecules can be carried out by a sequence of three reactions: making cDNA copies of selected RNAs, using the polymerase chain reaction to increase the copy number of each cDNA, and transcribing the cDNA copies to obtain RNA molecules having the same sequences as the selected RNAs. Any reaction or combination of reactions known in the art can be used as appropriate, including direct DNA replication, direct RNA amplification and the like, as will be recognized by those skilled in the art. The amplification method should result in the proportions of the amplified mixture being essentially representative of the proportions of different sequences in the mixture prior to amplification. It is known that many modifications to nucleic acids are compatible with enzymatic amplification. Modifications that are not compatible with amplification can be made after each round of amplification, if necessary.

"Randomized" is a term used to describe a segment of a nucleic acid having, in principle, any possible sequence over a given length. Randomized sequences will be of

various lengths, as desired, ranging from about eight to more than one hundred nucleotides. The chemical or enzymatic reactions by which random sequence segments are made may not yield mathematically random sequences due to unknown biases or nucleotide preferences that may exist. The term "randomized" is used instead of "random" to reflect the possibility of such deviations from non-ideality. In the techniques presently known, for example sequential chemical synthesis, large deviations are not known to occur. For short segments of 20 nucleotides or less, any minor bias that might exist would have negligible consequences. The longer the sequences of a single synthesis, the greater the effect of any bias.

A bias may be deliberately introduced into a randomized sequence, for example, by altering the molar ratios of precursor nucleoside (or deoxynucleoside) triphosphates in the synthesis reaction or the ratio of phosphoramidites in the chemical synthesis. A deliberate bias may be desired, for example, to affect secondary structure, to introduce bias toward molecules known to have facilitating activity, to introduce certain structural characteristics, or based on preliminary results.

In its most basic form, the SELEX process may be defined by the following series of steps:

1) A candidate mixture of nucleic acids of differing sequence is prepared. The candidate mixture generally includes regions of fixed sequences (i.e., each of the members of the candidate mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are selected either: (a) to assist in the amplification steps described below, (b) to mimic a sequence known to bind to the target, or (c) to enhance the concentration of a given structural arrangement of the nucleic acids in the candidate mixture. The randomized sequences can be totally randomized (i.e., the probability of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).

2) The candidate mixture is contacted with the selected target under conditions favorable for binding between the target and members of the candidate mixture. Under these circumstances, the interaction between the target and the nucleic acids of the candidate mixture can be considered as forming nucleic acid-target pairs between the target and those nucleic acids having the strongest affinity for the target.

3) The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with lesser affinity to the target. Because only an extremely small number of sequences (and possibly only one molecule of nucleic acid) corresponding to the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the nucleic acids in the candidate mixture (approximately 5-50%) are retained during partitioning.

4) Those nucleic acids selected during partitioning as having the relatively higher affinity to the target are then amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively higher affinity for the target.

5) By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer unique sequences, and the average degree of affinity of the nucleic acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a small number of unique nucleic acids representing those nucleic acids from the original candidate mixture having the highest affinity to the target molecule.

The SELEX Patent Applications describe and elaborate on this process in great detail. Included are targets that can be used in the process; methods for partitioning nucleic acids within a candidate mixture; and methods for amplifying partitioned nucleic acids to generate an enriched candidate mixture. The SELEX Patent Applications also describe ligands obtained to a number of target species, including both protein targets where the protein is and is not a nucleic acid binding protein.

SELEX provides high affinity ligands of a target molecule. This represents a singular achievement that is unprecedented in the field of nucleic acids research. The present invention applies the SELEX procedure to more complicated tissue targets.

Negative selection (Counter-SELEX) is optionally employed before, during or after the Tissue SELEX process. The negative selection provides the ability to discriminate between closely related but different tissue types. For example, negative selection can be introduced to identify nucleic acid ligands that have a high specificity for a tumor cell but do not recognize the cognate normal tissue. Similarly, nucleic acid ligands can be identified which specifically recognize atherosclerotic arterial tissue but not normal arterial tissue. Nucleic acid ligands which recognize fibrin, but not fibrinogen can also be identified by this method. Additionally, nucleic acid ligands to a cell type which express a

certain receptor can be counter-selected with a cell line engineered not to express the receptor (or other such macromolecule).

One of ordinary skill in the art will readily understand that various mechanisms can be employed to accomplish this negative selection. The following examples are provided mostly for illustrative purposes and are not meant in any way as limiting the procedures of negative selection. Negative selection or Counter-SELEX methods were first described in United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands that Discriminate Between Theophylline and Caffeine," now abandoned (see also United States Patent No. 5,580,737) which is herein incorporated by reference. A particular implementation of negative selection is embodied using equilibrium partitioning. In this method, two cell lines or other tissue types are separated by a semi-permeable membrane (0.45- 0.90 μm pore size) in an equilibrium dialysis chamber; one cell line is the neoplastic target cell line, the other, the normal tissue used for the negative selection. The choice of cell or tissue type for the negative selection will be determined by the specific end results desired and will sometimes consist of a non-malignant cell line of the same tissue type as the neoplastic target. For other experiments, various normal cell types could be combined to create the negative epitope "sink." The random pool of nucleic acids is placed into the dialysis chamber (on the side of the normal cells; this avoids background from high avidity targets which are common to both the tumor and normal cells) and allowed to equilibrate between the two cell lines. Those nucleic acid sequences that remain bound to the target cell line or tissue at equilibrium are selectively recovered and amplified for the next round of SELEX.

This example of negative selection methodology is quite powerful. First, equilibrium dialysis negative selection allows the positive and negative selection to be carried out *simultaneously*. Second, the stringency of the negative selection can be varied through the alteration of the relative amounts of "positive" and "negative" cells placed on each side of the dialysis membrane. These two characteristics of equilibrium dialysis negative selection allow precise control over the evolution of nucleic acid ligands specific for the target cell or tissue type.

This same type of equilibrium partitioning negative selection can be carried out with adherent cell lines. In this embodiment, monolayers of target and negative cells or tissues are plated in different wells of a multi-welled plate. After adherence, media, along

with an oligonucleotide pool, is added such that the wells are connected by the volume of cell media. After equilibration of the oligonucleotide pool, those sequences bound by the target cell line or tissue type would be isolated and amplified for the next round of SELEX.

The equilibrium negative selection strategies above offer a powerful way of generating nucleic acid ligands to tissue targets and especially tumor-associated antigens (TAAs).

Additionally, there are several other negative selection methods, which could be classified as "post-SELEX screening procedures." The simplest of these procedures is the testing of individual nucleic acid ligands (those sequences generated by tissue SELEX and demonstrated to be high-affinity ligands for the tissue target) against normal tissue for cross-reactivity. This approach, however, is a tedious and time-consuming process.

A more fruitful "post-SELEX" method is to perform a negative selection, for example using a normal tissue as the negative selection target, on a pool that has already been evolved from a SELEX against a desirable complex tissue target, for example a transformed cell line. This example would suggest the performance of two to three negative selections on a normal tissue using a late-round, highly evolved pool from a SELEX of a transformed cell line. The binding of certain sequences to the normal tissue would be used to subtract these sequences from the evolved pool. This method allows one to quickly eliminate from several hundred to several thousand nucleic acid sequences that show a high affinity for those targets common to both the normal and the transformed cell lines.

Another "post-SELEX" screening method is a variation of the photocrosslinking experiment described in Example 2 below. As an example, it is possible to synthetically incorporate a highly photoreactive nitrine group (which is also iodinatable) on the 5' end of a PCR primer used in the tissue SELEX protocols. Late-round pools from for example, a tumor cell line SELEX would be amplified with this photoactivatable (and ^{125}I-labeled) primer, and this sequence pool would then be irradiated in the presence of the tumor cell line, and in the presence of normal tissue. Membrane proteins would be isolated and solubilized for analysis on an SDS gel. One would expect to see many different protein epitopes tagged by specific oligonucleotide sequences, for both the tumor and the normal cell lines. A few tagged targets will be unique to the tumor cell line. Because the oligonucleotides have been photochemically linked to the protein targets in a manner which does not destroy the base sequence of the oligonucleotide, it is possible to isolate a

tumor-specific band from an SDS gel, and use PCR to recover a specific sequence motif that recognizes a particular tumor antigen. Thus, in one step, it is possible to remove from a pool these oligonucleotide sequences that recognize possibly hundreds of cell surface antigens, leaving one or a few families of sequences that bind specifically to a single tumor-specific antigen.

As described above, the Tissue SELEX methods can include the identification of macromolecules which comprise new epitopes on the tissue target. The nucleic acid ligand to the new epitope component of the macromolecule can be employed to purify, identify and characterize the macromolecule. The new macromolecule can be a previously unknown protein or peptide, lipid, carbohydrate, etc. Virtually any molecule that is part of the molecular make-up of a tissue can be identified by the Tissue SELEX process.

In order to fully exploit this aspect of the invention, it is important to develop strategies for the purification and identification of new macromolecules which comprise the new epitopes and to determine the roles these new macromolecular components of the tissue play in biological systems. The methods for purifying new macromolecules are well-known, especially in the art of protein purification. These standard purification methods include crosslinking, affinity chromatography, peptide microsequencing, Edman sequencing, mass spectrometry, and cDNA library searches.

The following discussion describes this process as it would be applied to the identification of a new tumor-associated antigen (TAA). For the purposes of this discussion, a TAA is a macromolecule that is expressed on a tumor cell, but not on a similar normal cell. A TAA may or may not be immunogenic. A TAA is merely one example of the kind of macromolecules which can be identified by the Tissue SELEX process and is simply used for illustrative purposes. However, it is readily apparent that this process can be extrapolated to any new macromolecule identified by the Tissue SELEX process.

As applied to TAAs, the identification of new TAAs by the Tissue SELEX process is composed of two main parts: one, developing strategies for the purification and identification of new TAAs, and two, the elucidation of the role these tumor antigens play in cancer (i.e., determining the biological significance of each particular TAA in the development and progression of a particular cancer).

The steps of purification and identification of most of the TAAs should be straightforward and understood by one skilled in the art of protein purification. As with

antibodies, SELEX provides a reagent -- a high-affinity ligand specific for the tumor antigen -- that is incredibly useful for the purification of the antigen from whole cells or other tissues. As a non-limiting example, most antigens will be amenable to some type of photo-affinity crosslinking or the negative selection strategies section above. Specific crosslinking of the TAA, using a photoactivatable oligonucleotide with a 3' biotin conjugate will allow one-pass purification of the TAA target using streptavidin coated beads. An alternative method to this purification strategy is to use a column-bound high-affinity nucleic acid ligand to affinity purify the TAA target from solubilized target cell membrane preparations.

There are many compelling reasons to believe that the method provided herein for identifying macromolecules that comprise new epitopes on tissues offers distinct advantages over traditional methods of new macromolecule discovery. Again, the following discussion will be directed to tumor-associated antigen discovery, but one will readily understand that it can be broadly extrapolated to all new macromolecule discovery.

As applied to tumor-associated antigens, one must fully consider that all that is known about tumor antigens has been derived from the immune system's reaction to particular antigens; science has depended on the particular restrictions of the immune system, and the system's repertoires to distinguish antigenic differences between neoplastic and normal tissue. It is entirely possible that other tumor antigens exist that are not subject to immune response. Some investigators have hypothesized that there may in fact be many antigenic differences between cancer and normal tissue, which are, unfortunately, not immunogenic.

The SELEX methodology provides an improved way to identify TAAs that avoids the restrictions posed by the immune system:

a. SELEX can actually provide a deeper search of TAAs than can the entire potential antibody repertoire of an organism -- the size of the nucleic acid libraries used in SELEX is unrivaled by any biological system;

b. SELEX provides nucleic acid ligands to targets, including those which are not antigenic to the immune system because of tolerance. Many of the TAAs which have been identified are oncofetal -- antigens expressed at some point during development or cell differentiation. As prior "self" antigens, they elicit no overt immune response because of earlier immune system tolerization. A SELEX-based search for TAAs avoids the circular

nature of using the immune system as a means of identifying tumor antigens;

c. SELEX nucleic acid ligands have been shown to be exquisitely sensitive to target conformation. While most antibodies recognize conformational, or discontinuous epitopes, antibody functional epitopes are composed of only a few amino acids. The potential binding surface of an oligonucleotide ligand is much larger than that of an antibody variable region, and may provide greater conformational discrimination of large targets. Additionally, cross-reactivity for SELEX ligands is substantially less of a problem than for monoclonal antibodies. A considerable set of restrictions also controls T-cell mediated tumor responses. These immune system limitations provide important biological functions; however, they limit the immune system's power for TAA identification.

d. SELEX is possibly more sensitive to small quantities of antigen than the immune system. Although the immune system's threshold for reactivity has been estimated to be 200 copies/cell for an antigenic MHC-presented peptide, a B-cell antibody response (necessary for any antigen that is not a peptide, i.e., carbohydrates, lipids or conformational antigens) to a monovalent target requires antigen concentrations of about 100 mM. SELEX can generate ligands to TAA targets with a low representation on the cell surface; and

e. SELEX provides a rapid and thorough method of TAA discovery. Screening of monoclonal antibodies to tissue sections, and purification and identification of MHC peptides are painstaking processes that set practical limits on the depth and completeness of searches for TAAs. Tissue SELEX experiments take a much abbreviated length of time.

Nucleic acid ligands to tissue targets or the tissue epitopes identified by the method of this invention are useful as diagnostic reagents, pharmaceuticals and as transportation escorts to target organs. The nucleic acid ligands are also useful for the identification of new macromolecules. The nucleic acid ligands are useful in any application that would be suitable for use of an antibody.

As diagnostic reagents, the ligands or tissue epitopes can be used in both *in vitro* diagnostics and *in vivo* imaging applications. The SELEX method generally, and the specific adaptations of the SELEX method taught and claimed herein specifically, are particularly suited for diagnostic applications. SELEX identifies nucleic acid ligands that are able to bind targets with high affinity and with surprising specificity. These characteristics are, of course, the desired properties one skilled in the art would seek for a

diagnostic ligand. Details regarding use of the ligands in diagnostic applications is well known to one of ordinary skill in the art. Nucleic acid ligands that bind specifically to pathological tissues such as tumors may have a role in imaging pathological conditions such as human tumor imaging and even therapeutic delivery of cytotoxic compounds or immune enhancing substances.

The nucleic acid ligands of the present invention may be routinely adapted for diagnostic purposes according to any number of techniques employed by those skilled in the art. Diagnostic agents need only be able to allow the user to identify the presence of a given target at a particular locale or concentration. Simply the ability to form binding pairs with the target may be sufficient to trigger a positive signal for diagnostic purposes. Those skilled in the art would also be able to adapt any nucleic acid ligand by procedures known in the art to incorporate a labelling tag in order to track the presence of a ligand. Such a tag could be used in a number of diagnostic procedures.

Specifically, oligonucleotide ligands with high specificity for particular tumor antigens could become as important as monoclonal antibodies for the detection, imaging, and surveillance of cancer. Modified nucleic acid ligands show nuclease resistance in plasma, and the use of 5' and 3' capping structures will provide stability in animals that rivals that of monoclonal antibodies (and without the immunogenicity of animal-derived MAbs). Radionuclides, magnetic compounds, and the like can be conjugated to tumor-specific oligonucleotides for cancer imaging. SELEX tumor ligands can also be used to determine if these tumor antigens are sloughed off tumors, and are detectable in the plasma like PSA.

The nucleic acid ligands to tissue targets or newly identified macromolecule components of tissue are also useful as pharmaceuticals. Therapeutic uses include the treatment or prevention of diseases or medical conditions in human patients. Therapeutic uses also include veterinary applications. The ligands can bind to receptors and be useful as receptor antagonists. Conversely, under certain circumstances the ligands can bind to receptors and cause receptor capping and act as receptor agonists.

In order to produce nucleic acids desirable for use as a pharmaceutical, it is preferred that the nucleic acid ligand (1) binds to the target in a manner capable of achieving the desired effect on the target; (2) be as small as possible to obtain the desired effect; (3) be as stable as possible; and (4) be a specific ligand to the chosen target. In most situations, it is preferred that the nucleic acid ligand have the highest possible affinity to the target.

One problem encountered in the therapeutic use of nucleic acids is that oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes such as endonucleases and exonucleases before the desired effect is manifest. Certain chemical modifications of the nucleic acid ligand can be made to increase the *in vivo* stability of the nucleic acid ligand or to enhance or to mediate the delivery of the nucleic acid ligand. Modifications of the nucleic acid ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

Where the nucleic acid ligands are derived by the SELEX method, the modifications can be pre- or post- SELEX modifications. Pre-SELEX modifications yield nucleic acid ligands with both specificity for it tissue target and improved *in vivo* stability. Post-SELEX modifications made to 2'-OH nucleic acid ligands can result in improved *in vivo* stability without adversely affecting the binding capacity of the nucleic acid ligands. The preferred modifications of the tissue nucleic acid ligands of the subject invention are 5' and 3' phosphorothioate capping and/or 3'-3' inverted phosphodiester linkage at the 3' end. In one preferred embodiment, the preferred modification of the tissue nucleic acid ligand is 3'-3' inverted phosphodiester linkage at the 3' end. Additional 2'-fluoro (2'-F) and/or, 2' amino (2'-NH_2), and/or 2'-O-methyl (2'-OMe) modification of some or all of the nucleotides is preferred.

Nucleic acid ligands to tissue targets or epitopes identified by the method of the subject invention are also useful as transporter-chaperones. Therapeutic uses include delivery of pharmaceuticals to target organs or tissues via ligand chaperones or ushers that recognize transporter molecules specific to or enhanced in a target organ. For example, delivery of pharmaceuticals to organs such as the brain can be enhanced by employment of nucleic acid ligands to transporter molecules in the blood brain barrier (BBB) cerebral endothelial tissue or the CSF-blood barrier endothelial tissue. In another embodiment, the

nucleic acid ligand may not only traverse the endothelial barrier, but may also itself act as a pharmaceutical in the brain. Additionally, use of the Tissue SELEX method for identification of transport molecules in target organs can significantly expand the number of transporters identified and characterized for that organ, thereby increasing the number of transport options for the target organ.

Standard formulations can be used for the nucleic acid ligands of the invention and are known to one of ordinary skill in the art.

The following example, which describes the identification of nucleic acid ligands to blood brain barrier and choroid plexus tissues, provides a non-limiting description of the present invention.

Example One

Nucleic Acid Ligands to Blood Brain Barrier and Choroid Plexus Tissues

This example describes a procedure to obtain nucleic acid ligands to target molecules present in the cerebral endothelium of the blood brain barrier (BBB) and the choroid plexus endothelium of the cerebral spinal fluid (CSF)-blood barrier.

Circulatory system access of pharmaceuticals to the brain is limited by the highly restricted permeability of the endothelial layer of the BBB. Limited diffusion of compounds across the BBB necessitates specific transport mechanisms for most nutrients and metabolites required for normal brain function. Therapeutic agents intended for use in treatment of CNS disorders must cross the BBB either by subversion of identified transporters with normal brain function or by diffusion through the endothelial layer. Utilization of either route limits the application of known compounds to CNS disease and imposes severe restrictions on the design of new agents (see Greig *et al.* (1995) in New Concepts of a Blood-Brain Barrier, London, Plenum Press; Neuwelt (1995) in New Concepts of a Blood-Brain Barrier, London, Plenum Press; and Tan *et al.* (1996) in Growth Factors as Drugs for Neurological and Sensory Disorders, London, John Wiley and Sons).

A second route of limited access to the brain is provided across the cerebrospinal fluid (CSF)-blood barrier. The ventricles of the brain are filled with CSF and the entire brain floats in the skull in a cushion of CSF. Thus, the CSF bathes the brain internally and externally, CSF is secreted by the epithelium of the choroid plexus into the ventricles in the center of the brain and from there the CSF flows down the spinal column and around the brain. The choroid

plexus is the site of both production and regulation of the composition of CSF and the choroid plexus epithelium contains a wide array of metabolic transporters, some of which are not found in the BBB. Therefore, transport systems unique to the choroid plexus can add to the array of identified transporters targeted for drug transport from blood to brain.

Most attempts to deliver compounds with known therapeutic potential across the BBB have followed one of three approaches (Johansson (1992) Prog. Brain Res. 91:171-175): modification of systemically active compounds to increase lipophilicity and diffusion across the BBB (Smith (1992) Adv. Exp. Med. Biol. 331:83); modification of known agents to increase transport by specific nutritional transport mechanisms, for example cationization to increase transport of the cationic peptide transporters (Wadhwani *et al.* (1992) J. Neurosci. Res. 32:407-414); and conjugation of therapeutic agents to antibodies directed against membrane transporters, for example the transferrin receptor (Friden *et al.* (1993) Science 259:373-377). A variety of BBB-associated molecules have been identified including transporters and receptors of metabolites (such as those for amino acids, glucose and LDL), ion channels, a BBB-specific Na^+ - K^+ ATPase, membrane-associated enzymes and surface antigens of unknown role identified by monoclonal antibodies and that are unique, or highly enriched in the BBB (reviewed in Englehardt and Risau (1995) in New Concepts of a Blood-Brain Barrier, Paris, Plenum). Direct application of drugs to the brain either by injection into the brain or by application to exposed and artificially permeabilized BBB have also been attempted, also with moderate and mixed success (Black *et al.* (1977) Neurosurg. 86:603-609). Recent, modestly successful attempts at gene transfer to the brain have been reported (Zlokovic and Apuzzo (1997) Neurosurgery 40:805-813).

To identify new compounds capable of traversing the BBB or the choroid plexus endothelium it would be advantageous to test large numbers of different molecules solely for their ability to cross the endothelium; ideally, no restriction to a specific transport mechanism would be imposed. In this way, molecules will be identified that traverse the BBB or choroid plexus by either known or unknown mechanisms. Successful compounds could subsequently be tested for efficacy and specificity *in vivo*. Two requirements for this approach are a large selection of compounds to be tested and an efficient initial method for screening them. SELEX provides the large range of compounds for testing and tissue-culture models of both the BBB and the CSF-blood barrier provide methods for initial screening of nucleic acid ligands for the ability to cross the BBB or CSF-blood barrier. Following initial screening *in vitro*, the compounds would be tested *in vivo* for CNS specificity and, if necessary, subjected to additional selection *in vivo*.

A. MATERIALS AND METHODS

BBB Tissue Culture.

Both primary cultures and established cerebral endothelium cell lines have been used as *in vitro* models of the BBB (reviewed in Joó (1993) Neurochem. Int. 23:499-521). Cultures have been established from a variety of animals including cows (Dehouck *et al.* (1990) J. Neurochem. 54:1798-1801), rats (Ichikawa *et al.* (1996) J. Pharm. Toxicol. Meth. 36:45-52) and humans (Golden *et al.*(1997) J. Clin. Invest. 99:14-18). Although specific methods vary, in general, these cultures are established from tissue preparations enriched for cerebral endothelial cells and co-cultured with astrocytes or astrocyte-conditioned medium. The astrocyte co-culture is required to establish and maintain the permeability characteristics of the BBB. Evidence that these cultures represent BBB include morphological, histological and biochemical criteria as well as the demonstration of very high electrical resistance. Although non-endothelial cells may be present in these cultures, a significant advantage of several culture methods is that the endothelial cells are maintained in a chamber separated from the feeder cells by a porous membrane (Figure 1). Endothelial cells grown in this way form tight junctions and exhibit cell polarity characteristic of the BBB. Therefore, Fig. 1 has a compartment equivalent to the luminal side of the BBB that is exposed to the systemic circulation, and an abluminal compartment, or the "brain side" of the BBB.

To identify nucleic acid ligands capable of crossing the BBB, pools of nucleic acid ligands can be placed in the luminal compartment and those that traverse the endothelial layer recovered in the abluminal compartment. Dehouck *et al.* ((1990) J. Neurochem. 54:1798-1801) demonstrated that by transferring the dish-insert containing the luminal compartment to successive abluminal wells, a time course of transfer across the cultured endothelium could be established. Those nucleic acid ligands that transit the BBB into the abluminal compartment will be recovered, amplified and retested.

The culture system shown in Fig. 1 has proven useful for modeling the BBB, however, the extent to which these cultures mimic the characteristics of the BBB *in vivo* is affected by a variety of parameters including medium composition, filter matrix and hydrostatic pressure (Wolburg *et al.* (1994) J. Cell Sci. 107:1347-1357; Stanness *et al.* (1996) NeuroToxicology 17:481-496). For example, the electrical resistance of the BBB is approximately 2000 Ω cm^2 (Crone and Olesen (1984) Brain Res. 241:49-55). Resistance across

the endothelial layer established in the static system shown in Fig. 1 was 700-800 Ω cm^2 (Dehouck *et al.* (1995) J. Neurochem. 54:1798-1801). When grown under dynamic conditions in an attempt to mimic luminal blood flow, the resistance of the cultured endothelium was 2900 Ω cm^2 (Stanness *et al.* (1996) NeuroToxicology 17:481-496). Therefore, although the culture method shown diagramed in Fig. 1 is simplest to establish and is useful for initial screening, more complex culture systems could be established if it becomes necessary or desirable to mimic specific characteristics of the BBB absent form the initial culture method.

Choroid Plexus Tissue Culture.

The choroid plexus is the primary site of the blood-CSF barrier. The CSF is produced by the epithelial cells of the choroid plexus and transported through the endothelial layer into the brain. In a culture system similar to that used for culturing cerebral endothelium, primary cultures of choroid plexus epithelium and endothelium have been established to study transport into the CSF. Choroid plexus culture has been established from rabbit (Ramanathan *et al.* (1996) Pharm. Res. 14:406-409) and pig (Gath *et al.* (1995) Cerebral Vascular Biology: Biology and Physiology of the Blood-Brain Barrier, Paris, Plenum; Hoffmann *et al.* (1996) J. Cell. Physiol. 169:235-241) and these cultures exhibit polarity and permeability characteristics of the blood-CSF barrier. Although not as extensively characterized and studied as *in vitro* models of the BBB, the choroid plexus model from rabbits has been demonstrated to permit transport of amino acids in the proper direction (Ramanathan *et al.* (1997) Pharm. Res. 13:952-956) and the cultured pig choroid plexus has been shown to produce fluid from the epithelial compartment with characteristics of CSF (Gath *et al.* (1995) Cerebral Vascular Biology: Biology and Physiology of the Blood-Brain Barrier, Paris, Plenum). Therefore, these cultures provide an opportunity to identify nucleic acid ligands that will be selectively transported into the CSF and with it, into the brain.

Cultures of pig choroid plexus are grown in two-chamber dishes as shown in Fig. 2 (Gath *et al.* (1995) Cerebral Vascular Biology: Biology and Physiology of the Blood-Brain Barrier, Paris, Plenum). By morphological and histological criteria, these cultures exhibit characteristics of choroid plexus with proper polarity. To identify nucleic acid ligands that can be transported into the CSF, pools of nucleic acid ligands can be added to the serum compartment in Fig. 2 and the *in vitro* CSF can be tested for presence of transported nucleic acid ligands. Those nucleic acid ligands appearing in the CSF will be recovered, amplified and retested.

In Vivo Selection.

Nucleic acid ligands selected for the ability to traverse the BBB or blood-CSF barrier using the *in vitro* cultures of the appropriate tissues can, if desired, be further selected using an *in vivo* selection procedure. An *in vivo* selection procedure can be used to select for nucleic acid ligand specificity for the blood brain or blood-CSF barriers. Tissue specificity is a desirable, although not essential, quality of potential therapeutic compounds.

An advantage of *in vitro* selection is the opportunity to use human tissue cultures in the initial stages. Following several rounds of *in vitro* selection, the pool of nucleic acid ligands able to traverse the *in vitro* barriers can be screened in rats or mice for the ability to traverse the barrier *in vivo*. By appropriate and established procedures the rate with which these molecules become concentrated in the brain can be determined. Simultaneously, the specificity of this localization can be assessed. Continued refinement of the pool will be achieved by recovery of nucleic acid ligands concentrated to the brain, amplification and retesting *in vivo*. Analyses using *in vivo* selection have demonstrated that the brain is not a tissue in which non-specific localization of nucleic acid ligands is problematic. Therefore, the signal-to-noise ratio in selection for localization to the brain should be very high.

B. THERAPEUTIC APPLICATION OF NUCLEIC ACID LIGANDS CAPABLE OF TRAVERSING THE BBB OR CSF-BLOOD BARRIER

In addition to identification of molecules capable of traversing the BBB or CSF-blood barrier, further selection of these compounds for specific therapeutic benefit is also contemplated. However, the molecules identified in the selections described can be useful in either of two applications: as direct therapeutic agents or as BBB or CSF-blood barrier chaperones for compounds with known therapeutic potential that are unable themselves to traverse the epithelia. Nucleic acid ligands with the ability to traverse the BBB or CSF-blood barrier and directly effect a beneficial therapeutic outcome are the ideal products of these screens. They are unlikely to predominate in the pool of molecules identified. Within this pool, however, it is likely that a subset will be identified that very efficiently traverse the cerebral or choroid plexus epithelia and that will be candidates to be used as escorts enabling the transport of other molecules with therapeutic activity. BBB or CSF-blood barrier escort nucleic acid ligands could be used to direct liposomes and facilitate their diffusion across the cerebral or choroid plexus endothelial barriers. As ushers of liposomes loaded with therapeutically active molecules, the escorts would permit

selective targeting of these compounds to the brain, preventing the potentially damaging side effects of inappropriate application of CNS-specific reagents to healthy tissues.

CLAIMS:

1. A method for identifying nucleic ligands to target molecules within a blood brain barrier or cerebral spinal fluid (CSF)-blood barrier tissue comprising:

a) preparing a candidate mixture of nucleic acids;

b) contacting said candidate mixture of nucleic acids with said blood brain barrier or CSF-blood barrier tissue, wherein nucleic acids having an increased affinity to the target molecules relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acid sequences with relatively higher affinity and specificity for binding to said target molecules, whereby nucleic acid ligands of said target molecules may be identified.

2. The method of claim 1 further comprising:

e) repeating steps b), c) and d).

3. The method of claim 1 wherein the tissue is selected from the group consisting of cerebral epithelial cells and choroid plexus epithelial cells.

4. The method of claim 1 wherein said candidate mixture is comprised of single-stranded nucleic acids.

5. The method of claim 4 wherein said single-stranded nucleic acids are ribonucleic acids.

6. The method of claim 4 wherein said single-stranded nucleic acids are deoxyribonucleic acids.

7. A nucleic acid ligand to a blood brain barrier or CSF-blood barrier molecule identified according to the method of claim 1.

8. A purified and isolated non-naturally occurring nucleic acid ligand to a blood brain barrier or CSF-blood barrier tissue.

9. The purified nucleic acid ligand of claim 8 which is a non-naturally occurring nucleic acid ligand having a specific binding affinity for a blood brain barrier or CSF-blood barrier molecule, such molecule being a three dimensional chemical structure other than a polynucleotide that binds to said nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein said nucleic acid ligand is not a nucleic acid having the know physiological function of being bound by the molecule.

10. The nucleic acid ligand of claim 8 which is a deoxyribonucleic acid ligand.

11. The nucleic acid ligand of claim 8 which is a ribonucleic acid ligand.

12. A method for identifying a target molecule of a blood brain barrier or CSF-blood barrier tissue comprising:

a) identifying a nucleic acid ligand to a new epitope of said target molecule by the method of claim 1;

b) purifying said target molecule of said blood brain barrier or CSF-blood barrier tissue away from the remainder of said tissue on the basis of affinity between said new epitope and said nucleic acid ligand; and

c) identifying said target molecule.

13. The method of claim 12 wherein said target molecule is selected from the group consisting of a protein, a lipid and a carbohydrate.

14. A purified macromolecule identified according to the method of claim 12.

15. The purified macromolecule of claim 12 which is selected from the group consisting of a protein, a lipid and a carbohydrate.

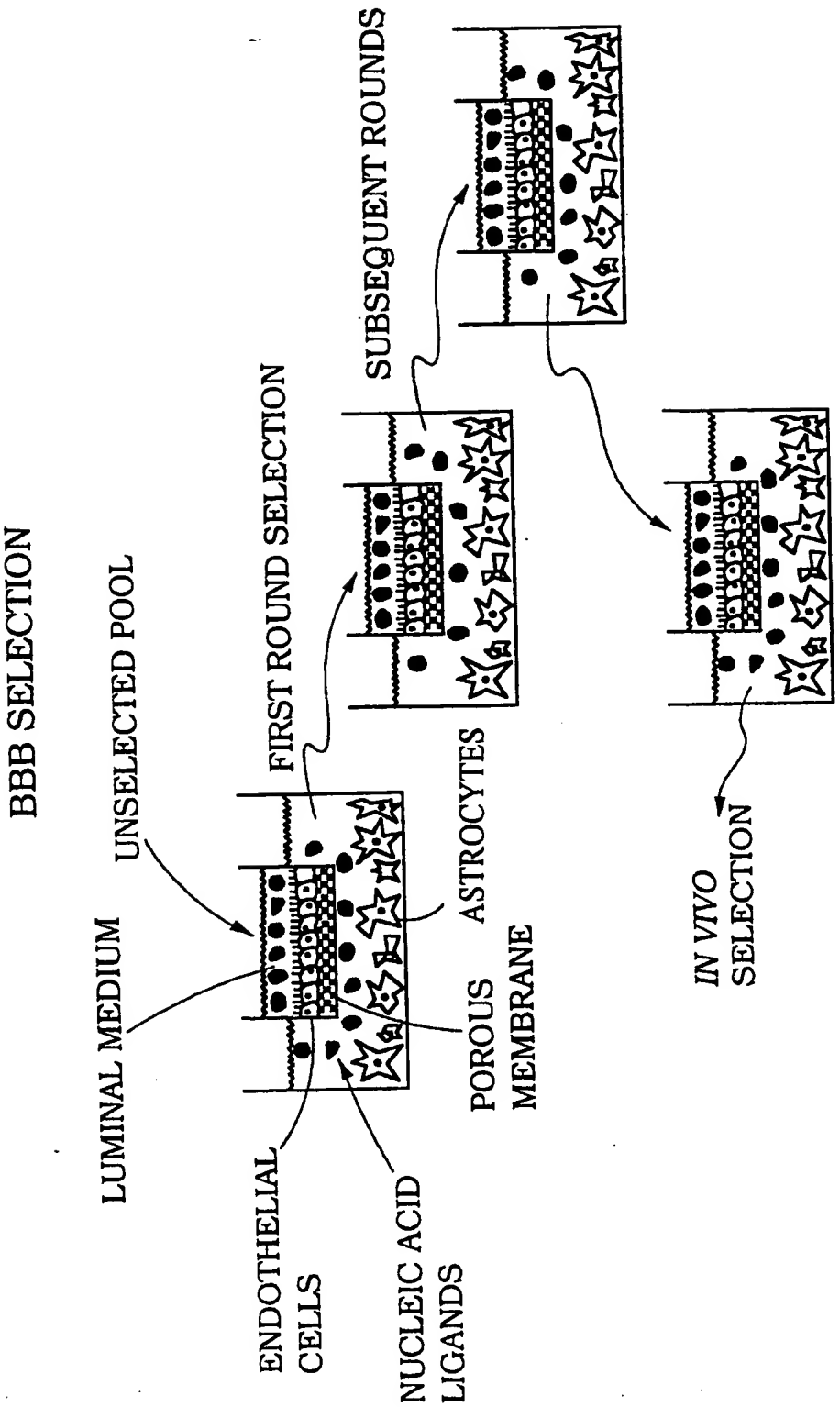
BBB SELECTION
UNSELECTED POOL
FIRST ROUND SELECTION
SUBSEQUENT ROUNDS
LUMINAL MEDIUM
ENDOTHELIAL CELLS
NUCLEIC ACID LIGANDS
POROUS MEMBRANE
ASTROCYTES
IN VIVO SELECTION

FIG. 1

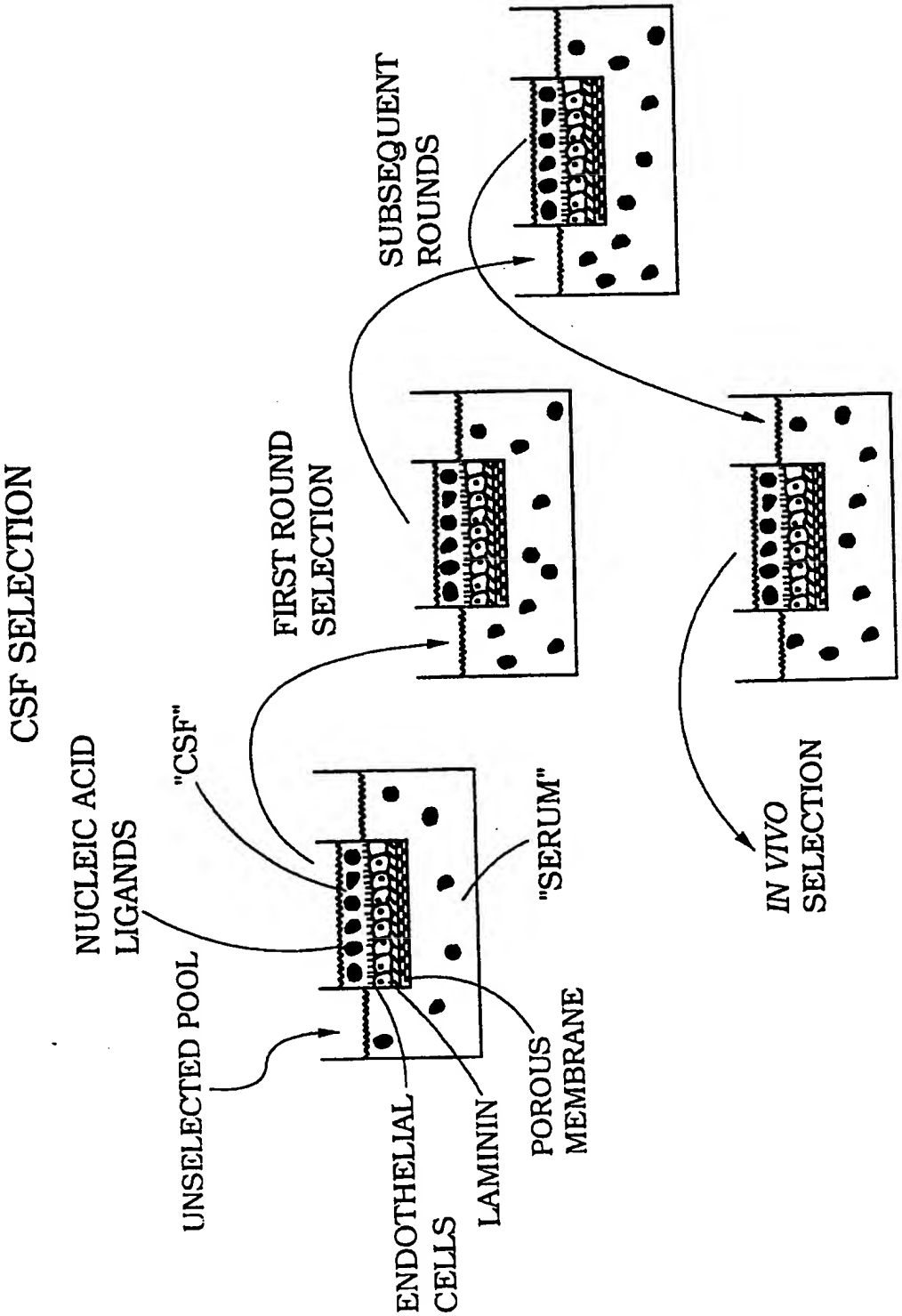
CSF SELECTION
NUCLEIC ACID LIGANDS
"CSF"
UNSELECTED POOL
ENDOTHELIAL CELLS
LAMININ
POROUS MEMBRANE
"SERUM"
FIRST ROUND SELECTION
SUBSEQUENT ROUNDS
IN VIVO SELECTION

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15619

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/02, 21/04; C12P 19/34; C12Q 1/68
US CL :435/6, 91.2; 536/24.3, 24.31, 25.4; 935/77, 78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/24.3, 24.31, 25.4; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG ONESEARCH: aptamer, nucleic acid ligand, tissue, blood brain barrier, CSF blood barrier

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92/14843 A1 (GILEAD SCIENCES, INC.) 03 September 1992, pages 57-58, 63-66, 97-98, 137, 140-143	1-13
Y	GATH et al. The Blood-CSF Barrier In Vitro, Biology and Physiology of the Blood-Brain Barrier , Couraud and Scherman, New York: Plenum Press, 1996, see entire document.	1-13
Y	DEHOUCK et al. Blood-Brain Barrier In Vitro, Biology and Physiology of the Blood-Brain Barrier, Couraud and Scherman, New York: Plenum Press, 1996, see entire document	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
28 OCTOBER 1998

Date of mailing of the international search report
17 DEC 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
STEPHANIE ZITOMER, PHD

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15619

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-13

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15619

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-13, drawn to a single inventive concept encompassing nucleic acid ligands to blood-brain barrier or CSF- blood barrier tissue, a method for preparing them and a method for using them
Group II, claim(s) 14 and 15, drawn to a purified macromolecule identified using a ligand of Group I.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because PCT Rule 13 does not provide for multiple products in an inventive group.